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Award Number: DAMD17-02-1-0343

TITLE: Whole Genome Epigenetics

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REPORT DATE: June 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2004	Annual Summary (1 May 2003 - 30 Apr 2004)

4. TITLE AND SUBTITLE
Whole Genome Epigenetics

5. FUNDING NUMBERS
DAMD17-02-1-0343

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

An understanding of the cancer cell begins with knowledge of the genetic alterations that lead to neoplastic transformation. Much progress has been made in identifying areas of deletion, amplification, and mutation in tumors. However, this is only part of the picture. Increasingly, we are learning that epigenetic changes, that is, changes in chromatin structure, are critically important in regulating cellular gene expression.

Recently, several labs have published manuscripts identifying RNA interference as being crucial for the establishment of such epigenetic changes in species as diverse as *Drosophila*, plants, and the fission yeast *S. pombe*. This knowledge presented a fantastic opportunity not only to stud epigenetic changes, but to actually selectively *create* epigenetic changes by creating germline transgenic mice in which a target gene has been silenced by RNAi.

After the demonstration that RNAi in mammalian cells an be mediated by vectors encoding short, hairpin RNAs (shRNAs), we sought to develop a system by which to create transgenic mice using this technology. We demonstrate that a stable, heritable RNAi trigger in the form of a short hairpin was successfully passed through the mouse germline. As well as demonstrating a technique that can be applied to any gene of interest, we have created gene knock-down mouse models for the Neil-1 DNA glycosylase involved in DNA repair pathways, and for p53. These observations open the way to the use of RNAi as a complement to standard knock-out methodologies and provides a means to rapidly assess the consequences of suppressing a gene of interest in a living animal. The vision dirving the creation of this technology was one of eventual RNAi-based therapeutics. One could particularly imagine silencing oncogenes in cancer cells to attenuate their tumorigenic tendencies.

14. SUBJECT TERMS
Epigenetics, microarray, methylation, RNAi

15. NUMBER OF PAGES
9

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction:

An understanding of the cancer cell begins with knowledge of the genetic alterations that lead to neoplastic transformation. Much progress has been made in identifying areas of deletion, amplification and mutation in tumors. However, this is only part of the picture. Increasingly, we are learning that epigenetic changes are critically important in regulating cellular gene expression.

Epigenetic is defined as a change in gene expression that is heritable in *cis* with a particular allele but that is not specified by an alteration in the DNA sequence of the gene. One mechanistic basis of epigenetic inheritance in mammals is chemical modification of the DNA, specifically the addition of methyl groups that correlate with changes in chromatin structure, and consequently gene expression.

My interest at the time of the writing of this proposal was to examine such epigenetic changes occurring in cancer cells. The eventual goal was the creation of a screening method for identifying, on a patient-to-patient basis, the epigenetic lesions in a particular tumor. This type of technology would allow the clinician to tailor treatment regimens on a per patient basis.

We now know that RNA interference (RNAi) is crucial for the establishment of such epigenetic changes in species as diverse as Drosophila, plants, and the fission yeast S. pombe. We also now know through work in our lab and others that RNAi is functional in mammals. Through as yet unknown mechanisms, RNAi based technologies can control expression of a gene of interest. It is becoming increasingly likely that RNAi is involved in regulation of heterochromatin (the basis of the epigenome) in mammals. Last year I embarked on a study to create germline transgenic mice in which a target gene has been silenced by RNAi. The vision driving the creation of this technology was one of RNAi-based therapeutics. One could particularly imagine silencing oncogenes in cancer cells to attenuate their tumorigenic tendencies.

Body:

After the demonstration that RNAi in mammalian cells can be mediated by vectors encoding short, hairpin RNAs (shRNAs)[1], we sought to develop a system by which to create transgenic mice using this technology. Last year we demonstrated that a stable, heritable RNAi trigger in the form of a short hairpin was successfully passed through the mouse germline[2]. We observed specific suppression of a target gene at the level of mRNA and protein in multiple tissues. These observations open the way to the use of RNAi as a complement to standard knock-out methodologies and provides a means to rapidly assess the consequences of suppressing a gene of interest in a living animal.

In addition to the Neil-1 knockdown mice, we have created p53-deficient mice through the use of the same hairpin-based technology. The p53 hairpins that I have used have been validated by Hemann et al.[3], and shown to produce distinct tumor types *in vivo* when used in the context of reconstituted bone marrow. These p53-knockdown transgenic mice are currently being characterized. Figure 1 shows western blot confirmation of the function of the hairpin using an antibody to p53 and a control antibody to tubulin. As seen below, fibroblast cell lines created from non-transgenic animals are able to induce p53 expression in response to the DNA-damaging drug adriamycin. Transgenic animals carrying a short hairpin to p53 (shp53C) are unable to induce p53 protein expression, indicating that the hairpin is functional.

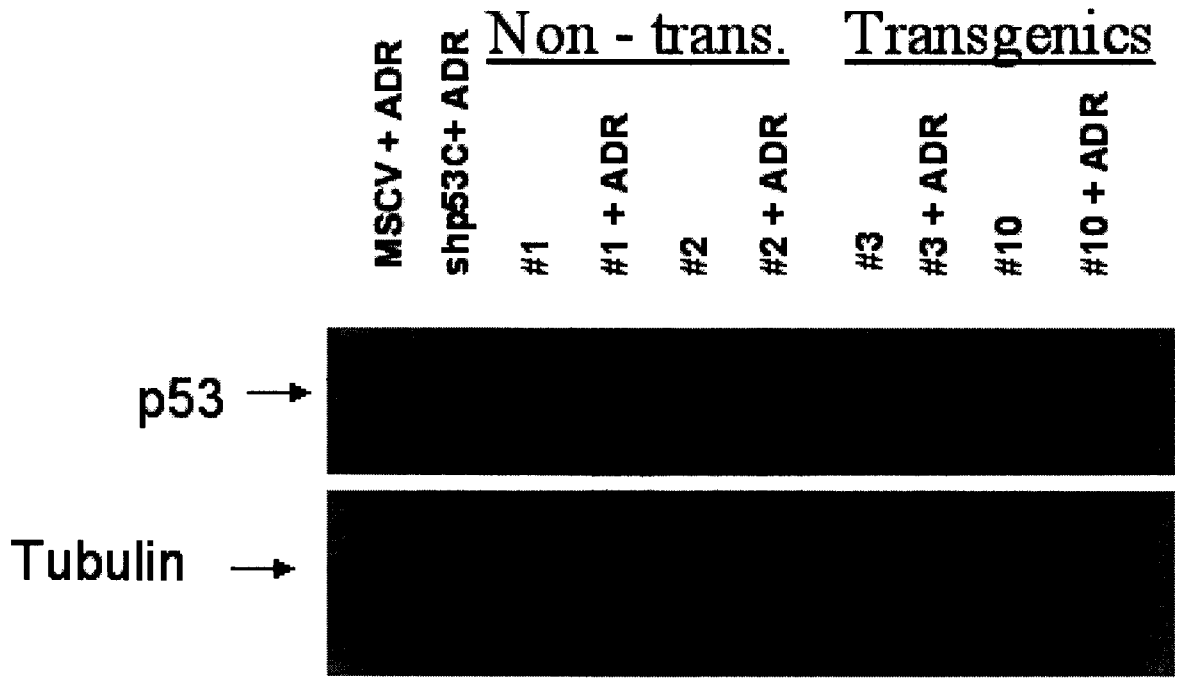

Figure 1: Transgenic mice carrying a hairpin to p53 are unable to induce p53 protein expression in the presence of a DNA-damaging agent (adriamycin= ADR). Western blot of fibroblast cell lines with p53 antiserum. Numbers 1 and 2 are non-transgenic pups, and numbers 3 and 10 are their transgenic littermates. The MSCV +ADR and shp53+ADR are wildtype MEFs infected with a virus either empty (MSCV) or MSCV carrying the hairpin. This control shows that the hairpin is able to knock-down p53 expression in another context.

To date, progress on this project has been a bit slow. When we transferred the mice from Stony Brook University (where the transgenics were made) to Cold Spring Harbor Laboratory (where we wish to do the study), they had to remain in quarantine for 6 months. According to lab policy, breeding is not allowed in quarantine. In addition, we have discovered that the male mice carrying the transgene are sterile. This is to be expected if the hairpin does indeed recapitulate the phenotype of p53 null mice *in vivo*.

P53 null males are sterile due to disruption of proper apoptosis in the testes. This phenotype also confirms that our hairpin acts as a dominant, trans-acting signal *in vivo*, as one copy of the transgene is sufficient to produce the sterility phenotype in the males. This phenotype will be expected to slow progress, as we will have to propagate the colony exclusively through females. The mice are currently being monitored for the development of tumors.

Key Research Accomplishments:

- Using the p53 knockdown mice for further confirmation of the efficacy of a generally applicable technique for creating germline transgenic mice in which a target gene has been silenced by RNAi

Reportable Outcomes:

Manuscripts:

Carmell MA and Hannon GJ. RNAseIII enzymes and the initiation of gene silencing. Nat Struct Mol Biol. 2004 Mar; 11(3): 214-218.

Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, Elledge SJ, Anderson KV, Hannon GJ. Dicer is essential for mouse development. Nat Genet 2003 Nov; 35(3): 215-217

Book Chapter:

"Achieving Stable, heritable gene silencing in the mouse." M. Carmell and G. Hannon in "RNA interference (RNAi): Nuts and Bolts of RNAi technology. D.R. Engelke, ed. Chapter 11, pages 217-234.

Presentations:

New York Academy of Sciences, RNAi Discussion Group, May 2004, Featured speaker.

Conclusions:

I have been working on further characterization of the p53 knockdown mouse lines. Progress has slowed due to quarantine of the mice, but is expected to pick up soon. Perhaps the true test of the ability of the hairpin to recapitulate the p53 phenotype *in vivo* will be tumorigenesis. I am currently waiting to see if the mice will develop tumors.

References:

1. Paddison, PJ, et al. Genes Dev. 2002 Apr 15;16(8):948-58.

2. Carmell, MA, et al. Nat Struct Biol. 2003 Feb; 10(2): 12-13.

3. Hemann, MT, et al. Nat Genet. 2003 Mar;33(3):396-400.